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The molecular chaperone SecB is released from the carboxy-terminus of SecA during initiation of precursor protein translocation

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The chaperone SecB keeps precursor proteins in a translocation-competent state and targets them to SecA at the translocation sites in the cytoplasmic membrane of *Escherichia coli*. SecA is thought to recognize SecB via its carboxy-terminus. To determine the minimal requirement for a SecB-binding site, fusion proteins were created between glutathione-*S*-transferase and different parts of the carboxy-terminus of SecA and analysed for SecB binding. A strikingly short amino acid sequence corresponding to only the most distal 22 aminoacyl residues of SecA suffices for the authentic binding of SecB or the SecB–precursor protein complex. SecAN880, a deletion mutant that lacks this highly conserved domain, still supports precursor protein translocation but is unable to bind SecB. Heterodimers of wild-type SecA and SecAN880 are defective in SecB binding, demonstrating that both carboxy-termini of the SecA dimer are needed to form a genuine SecB-binding site. SecB is released from the translocase at a very early stage in protein translocation when the membrane-bound SecA binds ATP to initiate translocation. It is concluded that the SecB-binding site on SecA is confined to the extreme carboxy-terminus of the SecA dimer, and that SecB is released from this site at the onset of translocation.

Keywords: chaperone/SecA/SecB/secretion

Introduction

In *Escherichia coli*, the translocation of precursor proteins across the cytoplasmic membrane is catalysed by the translocase, a multi-subunit membrane protein enzyme (Wickner *et al.*, 1991; Driessen, 1994). The core of this complex is a heterotrimeric integral domain with the SecY, SecE and SecG polypeptides as subunits, and SecA as peripheral component (Brundage *et al.*, 1990; Nishiyama *et al.*, 1994; Douville *et al.*, 1995). The SecYEG complex serves as the high-affinity binding site for SecA (Hartl *et al.*, 1990). SecD and SecF are integral membrane proteins that are not essential for precursor protein translocation *per se* but, when overproduced, stabilize the SecYEG-bound SecA in a membrane-inserted state (Kim *et al.*, 1994; Economou *et al.*, 1995). SecA is a large (M_r of 102 kDa), dimeric protein with many binding sites, conformations and activities (Driessen, 1994). In the cytoplasm, SecA functions as its own translational repressor (Dolan and Oliver, 1991). When bound to the membrane at SecYEG, SecA is 'activated' for high-affinity recognition of the SecB export chaperone (Hartl *et al.*, 1990), for the leader (signal) region of preproteins and for the mature domain of precursor proteins. Binding of the precursor protein activates SecA for the hydrolysis of ATP at one of its two ATP-binding sites (Lill *et al.*, 1990). The energy of ATP binding drives the insertion of a 30 kDa domain of SecA (Economou and Wickner, 1994) plus a loop of the signal sequence and the amino-terminal region of the preprotein across the membrane (Schiebel *et al.*, 1991). After insertion, the release of the inserted precursor protein requires hydrolysis of the ATP, and de-insertion of SecA occurs upon the binding and hydrolysis of a second ATP at a distinct ATP-binding site on the SecA molecule (Economou *et al.*, 1995). After de-insertion, SecA is free to exchange with cytosolic SecA, while protonmotive force-driven translocation can occur at this stage, in which the precursor protein is not bound by SecA (Schiebel *et al.*, 1991; Driessen, 1992). SecA can re-engage the SecYEG-bound preprotein, driving the translocation of a discrete number of aminoacyl residues of the preprotein during a cycle of ATP binding and hydrolysis (Schiebel *et al.*, 1991; Arkowitz *et al.*, 1993; Uchida *et al.*, 1995).

Although detailed information is available on the reaction cycle of SecA-mediated preprotein translocation, little is known about the timing of the SecB–SecA association and dissociation reactions. SecB is a tetramer (Smith *et al.*, 1996) of identical subunits (M_r of 17 kDa) that functions as an export-dedicated chaperone (Kumamoto, 1991). It binds to a subset of nascent secretory proteins while they emerge from the ribosome (Kumamoto and Francetić, 1993; Randall *et al.*, 1997). In the cytosol, it acts as a true folding catalyst as it uses the energy of polypeptide binding to stabilize the precursor protein in an unfolded, non-aggregated state (Lecker *et al.*, 1989; Zahn *et al.*, 1996). SecB targets precursor proteins to the membrane surface by specifically binding to the SecA subunit of the translocase. The SecA–SecB interaction is weak in solution (Hoffschulte *et al.*, 1994), but occurs with high affinity at the membrane surface, and is promoted by precursor proteins (Hartl *et al.*, 1990). Although it appears that SecB is not necessary for translocation once it has targeted the precursor protein to the SecA subunit of the translocase, the question of at what stage SecB is released has not been addressed.

Recently, we have reported that the SecYEG-bound membrane-integrated form of SecA exposes a trypsin cleavage site to the periplasmic side of the membrane (Van der Does *et al.*, 1996). This site is on the carboxy-terminal flanking region of a fragment that has been identified as the 30 kDa SecA membrane-penetrating

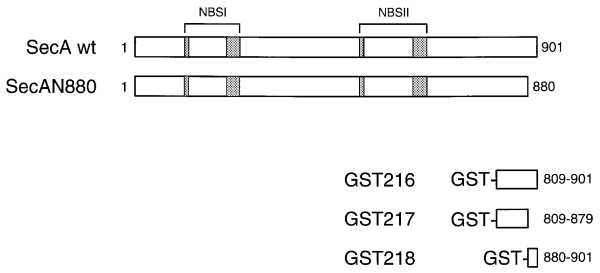

Fig. 1. Schematic representation of the truncated SecA proteins and the GST fusion proteins. SecAN880 is a SecA truncate which lacks the last 21 amino acid residues due to the introduction of a stop codon at position 881. The nucleotide-binding sites (NBS) are depicted as grey boxes, with NBSI and II as the high- and low-affinity binding site, respectively. GST of *Schistosoma japonicum* was amino-terminally fused to amino acids 809–901, 809–879 and 880–901 of wild-type SecA to produce GST216, GST217 and GST218, respectively.

domain (Price *et al.*, 1996). Other studies have shown that deletion of the carboxy-terminal 66–70 amino acids of SecA functionally destroys the SecB–SecA interaction, whereas it does not affect the ability of SecA to support SecB-independent translocation of preproteins (Rajapandi and Oliver, 1994; Breukink *et al.*, 1995). These data suggest that the carboxy-terminal region of SecA may have a dual function, i.e. SecB interaction and membrane integration. To determine what part of this region is the genuine SecB-binding domain, fusion proteins were constructed between glutathione-*S*-transferase (GST) and different parts of the carboxy-terminus of SecA. A highly conserved portion of SecA that consists of only the carboxy-terminal 22 amino acids and which is not part of the membrane-integrating region, suffices to constitute an authentic SecB-binding site. Further studies demonstrate that SecA releases SecB from its carboxy-terminus at the stage at which it inserts into the membrane. These studies assign the dual function of the SecA carboxy-terminal region to two distinct domains, and couple the SecB–SecA interaction to the nucleotide-modulated catalytic cycle of SecA that allows the stepwise translocation of precursor proteins across the membrane.

Results

SecB interacts with peptides derived from the carboxy-terminus of SecA

Deletion analysis of the SecA protein suggests that the SecB-interacting domain is contained in the carboxy-terminal 70 amino acids of SecA (Breukink *et al.*, 1995). To establish whether this region indeed harbours the SecB-binding domain, and to define its minimal size, *Schistosoma japonicum* GST was fused with different parts of the carboxy-terminus of SecA (Figure 1). GST216, GST217 and GST218 represent fusions between GST and the SecA amino acids 809–901, 809–879 and 880–901, respectively. Fusion proteins were purified by glutathione–agarose affinity chromatography (Figure 2A), and the presence of the extreme carboxy-terminus of SecA was verified with a polyclonal antibody raised against a synthetic peptide corresponding to amino acids 880–899 of SecA. This antibody recognizes the intact SecA (Van der Does *et al.*, 1996; data not shown) and the GST216 and GST218 fusion proteins (Figure 2B, lanes 2 and 4),

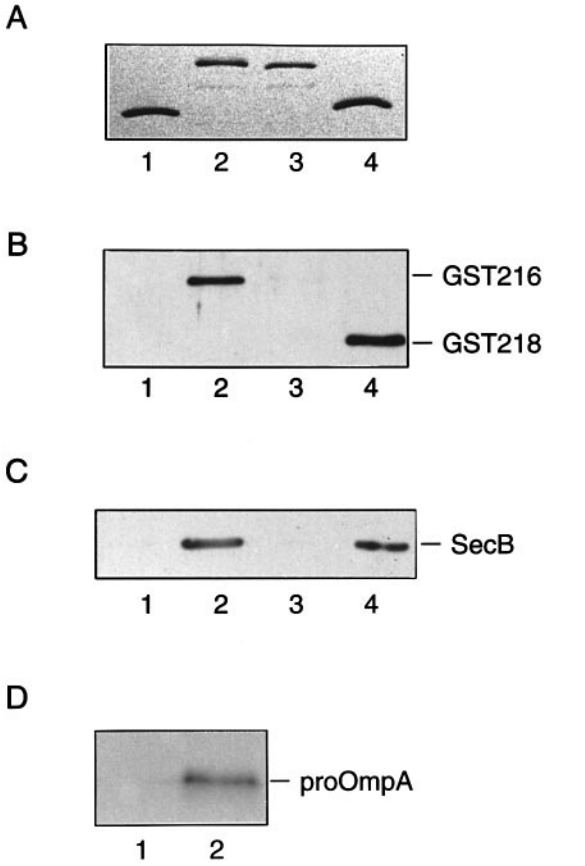

Fig. 2. SecB recognizes the carboxy-terminus of SecA. (**A**) Coomassie brilliant blue staining of purified GST (lane 1) and the GST fusion proteins GST216 (lane 2), GST217 (lane 3) and GST218 (lane 4). (**B**) Immunodetection of the purified GST fusion proteins with pAb 1045. pAb 1045 recognizes only GST216 and GST218 (lanes 2 and 4), while it fails to recognize GST and GST217 (lanes 1 and 3). (**C**) SecB binds to the extreme carboxy-terminus of SecA. GST (lane 1) and the fusion proteins GST216 (lane 2), GST217 (lane 3) and GST218 (lane 4) (5 μg each) were incubated with SecB (10 μg) and glutathione-coupled agarose beads in 200 μl of buffer C. After several washing steps with buffer C, the GST proteins were eluted from the beads with reduced glutathione. The presence of SecB in the eluent was checked with an antibody against SecB after SDS–PAGE and blotting onto PVDF. (**D**) Ternary complex formation between GST218, SecB and proOmpA. ^{35}S-labelled proOmpA was incubated with 10 μg of SecB and 5 μg of GST (lane 1) or 5 μg of GST218 (lane 2), and with 10 μl of glutathione-coupled agarose beads in 200 μl of buffer C. After several washing steps with buffer C, the GST proteins were eluted from the beads with reduced glutathione and the eluents were analysed by SDS–PAGE and autoradiography.

whereas wild-type GST and GST217 are not recognized (Figure 2B, lanes 1 and 3). The latter proteins lack the extreme carboxy-terminus of SecA. The interaction between the purified GST fusion proteins and SecB was monitored in a binding assay where GST and SecB were first pre-mixed and then supplemented with glutathione-coupled agarose beads. After several washing steps, the GST was eluted from the agarose beads with reduced glutathione. SecB was found to co-elute with the GST216 and GST218 proteins (Figure 2C, lanes 2 and 4), whereas no co-elution was observed with wild-type GST and GST217 (Figure 2C, lanes 1 and 3). These data indicate that SecB interacts with a peptide corresponding to the extreme carboxy-terminus of SecA.

To exclude the possibility that SecB binds to the GST

Table I. SecA- and SecB-dependent binding to urea-treated inner membrane vesicles

Ligand	IMV	Temperature (°C)	proOmpA	K_d[a] (nM)	B_{max}[b] (pmol/mg of protein)
SecB	wt	0	−	35	15
		0	+	15	15
	YEG$^+$	0	−	30 ± 5	115 ± 15
		0	+	10 ± 2	111 ± 15
		37	−	100	110
		37	+	60	110
SecA	YEG$^+$	0	−	7 ± 2	120 ± 20

[a]Data shown are of at least two independent experiments and, where indicated, the standard error of the mean is of four or more experiments.
[b]The maximum number of binding sites (B_{max}) for SecA and SecB are calculated assuming that SecA is a dimer and SecB is a tetramer, respectively.

fusion proteins because the SecA part is in an unfolded state and therefore recognized by SecB as a substrate, reduced bovine pancreatic trypsin inhibitor (R-BPTI) was included in the binding assay. R-BPTI is a high-affinity model substrate for the interaction between SecB and preproteins (Hardy and Randall, 1991; Fekkes *et al.*, 1995). Excess R-BPTI could not compete with the fusion proteins for the interaction with SecB (data not shown). Moreover, a ternary complex between proOmpA, the precursor form of the *E.coli* outer membrane protein A, SecB and GST218 could be formed (Figure 2D, lane 2), whereas GST did not bind proOmpA complexed to SecB (Figure 2D, lane 1). In the absence of SecB, there was no binding of proOmpA to GST or GST218 (data not shown). This shows that the carboxy-terminal domain of SecA interacts with a region of SecB that is different from the precursor protein-binding site, and hence mimics a binding site for a binary SecB–precursor protein complex.

The extreme carboxy-terminus of SecA is an authentic SecB-binding domain

To establish whether the carboxy-terminal peptide corresponding to amino acids 880–901 of SecA constitutes an authentic SecB-binding domain, competition experiments between GST218 and SecA for SecB binding were performed. For this purpose, membranes were used that were isolated from an *E.coli* strain that overproduces the SecYEG complex (termed SecYEG$^+$) (Van der Does *et al.*, 1996). Overproduction of the SecYEG complex results in an increased number of high-affinity binding sites for SecA (Douville *et al.*, 1995). Scatchard analysis (Scatchard, 1949) of the SecA-dependent binding of [^{125}I]SecB to the membrane demonstrates an almost 8-fold increase in the number (B_{max}) of high affinity SecB-binding sites (K_d of ~30 nM) upon SecYEG overproduction (Table I). When increasing concentrations of GST218 were included in the binding assay, the membrane-bound SecB was readily released, whereas the addition of wild-type GST was without effect (Figure 3). This phenomenon was specific for the SecA-dependent binding of SecB to the inner membranes as GST and GST218 had no influence on the low-affinity membrane binding of SecB, i.e. SecB binding in the absence of SecA (data not shown). These data indicate that the extreme carboxy-terminus of SecA fused to GST functions as an authentic SecB-binding site that competes with membrane-bound SecA for SecB binding.

To demonstrate further that the carboxy-terminal

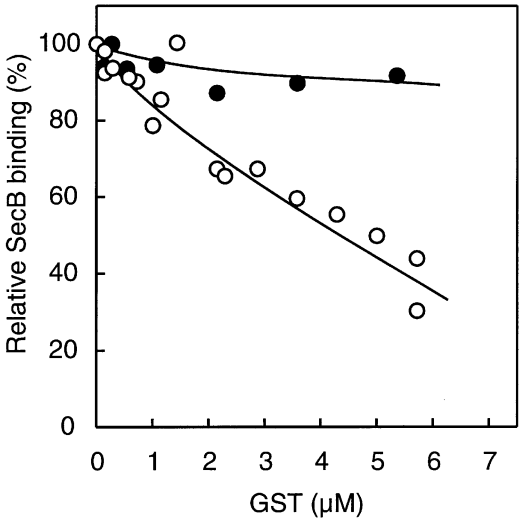

Fig. 3. GST218 competes with SecA for SecB binding. Urea-treated membranes (250 μg/ml) from SecYEG$^+$ cells were incubated on ice with 100 nM SecA and 10 nM ^{125}I-labelled SecB and increasing amounts (0.01–5.5 μM) of either GST (●) or GST218 (○) in 100 μl of buffer B. After 15 min, the membranes were sedimented through a 100 μl 0.2 M sucrose cushion and the amount of SecB in the pellet and supernatant was quantified in a γ-counter.

21 amino acids of SecA function as a SecB-binding site, a carboxy-terminal truncate of SecA was constructed, i.e. SecAN880, by the introduction of a stop codon in the *secA* gene at position 881. SecAN880 was able to functionally complement the SecA deficiency of *E.coli* strain MM66 (*geneX^{am}*, *supF^{ts}*) (Oliver and Beckwith, 1981) on rich medium at the non-permissive temperature (data not shown). This demonstrates that the extreme carboxy-terminus of SecA is not needed for viability under these conditions. The truncated SecA protein was purified from an overproducing strain, and tested for its ability to support the ATP-dependent translocation of proOmpA into urea-treated inner membranes (Figure 4A). In the absence of SecB, wild-type SecA and SecAN880 were nearly equally efficient in supporting translocation (lanes 2 and 3). On the other hand, the presence of SecB stimulated translocation with wild-type SecA (lane 4), whereas the stimulation was far less with SecAN880 (lane 5). Moreover, proOmpA that had been pre-incubated with SecB for 30 min was only poorly translocated when the SecAN880 was present (lane 7), while efficient translocation was observed with the wild-type SecA (lane 6). The

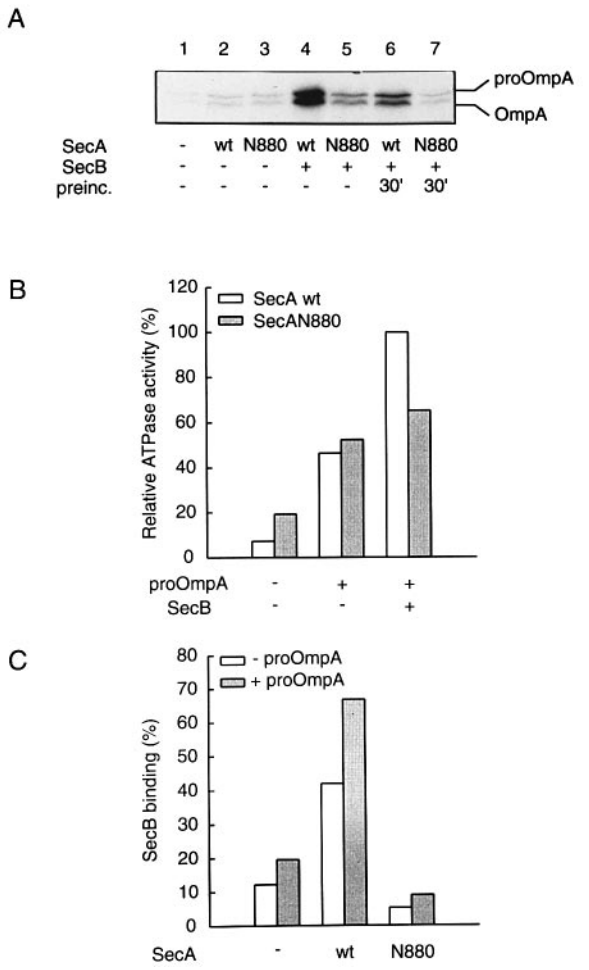

Fig. 4. SecAN880 is defective in the SecB interaction. (**A**) SecB-dependent preprotein translocation. Translocation of [^{35}S]proOmpA across urea-treated membranes from wild-type cells in the absence (lanes 1–3) or presence (lanes 4–7) of SecB was performed in the absence (lane 1) or presence of wild-type SecA (lanes 2, 4 and 6) or SecAN880 (lanes 3, 5 and 7) as described in Materials and methods. ProOmpA was either translocated directly (lanes 1–5) or first pre-incubated for 30 min with SecB (lanes 6 and 7), prior to the addition of IMVs. (**B**) SecA translocation ATPase activity. The ATPase activity of SecA (white bars) and SecAN880 (grey bars) was measured in the absence of proOmpA and SecB, in the presence of proOmpA alone or in the presence of proOmpA and SecB. (**C**) SecA-dependent SecB binding to membranes. [^{125}I]SecB (10 nM) was incubated with urea-treated membranes (250 μg/ml) derived from SecYEG$^+$ cells either in the absence (white bars) or presence (grey bars) of proOmpA (300 nM). The mixture was supplemented with 500 nM wild-type SecA (wt) or SecAN880 (N880), and the membrane binding of SecB was quantified as described in the legend to Figure 3.

observed stimulation by SecB of the protein translocation in the presence of SecAN880 is most likely a result of keeping proOmpA translocation-competent (Weiss *et al.*, 1988; Lecker *et al.*, 1990). These data indicate that the SecB-dependent targeting of proOmpA to SecAN880 is severely compromised. A similar phenomenon was observed when the translocation ATPase activity of SecA and SecAN880 was compared (Figure 4B). In the presence of urea-treated inner membrane vesicles (IMVs), the endogenous ATPase activity of SecAN880 was somewhat elevated as compared with that of wild-type SecA. Subsequent addition of proOmpA stimulated the ATPase activity of both proteins to similar extents, but SecB failed

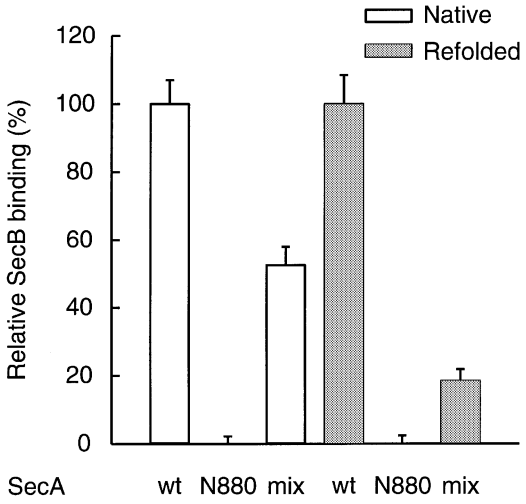

Fig. 5. Both carboxy-termini of the SecA dimer are needed for SecB binding. The binding of [^{125}I]SecB (10 nM) to urea-treated membranes of SecYEG$^+$ cells was measured in the presence of either native (white bars) or refolded (grey bars) SecA at a concentration of 50 nM, using either the wild-type SecA (wt), SecAN880 (N880) or a one-to-one mixture of these proteins (mix). The SecB binding with native wild-type SecA was set to 100%. Binding experiments were performed as described in the legend to Figure 3.

to stimulate further the ATPase activity of SecAN880 whereas an almost 2-fold increase was observed with the wild-type SecA. Finally, SecAN880 was analysed for its ability to bind ^{125}I-labelled SecB. For this purpose, SecYEG$^+$ IMVs were used (Figure 4C). After urea treatment, some SecA remains bound to the membranes (Van der Does *et al.*, 1996), giving rise to background binding of SecB in the absence of added SecA (Figure 4C). Addition of wild-type SecA resulted in a dramatic increase in the SecB binding, that was stimulated even further by the addition of proOmpA (Figure 4C), but not by OmpA (Hartl *et al.*, 1990; data not shown). In contrast, addition of SecAN880 resulted in a decreased level of SecB binding, whereas proOmpA was without any effect. Taken together, these data provide functional evidence that the extreme carboxy-terminus of SecA constitutes the SecB-binding site.

SecB binds the dimeric form of the carboxy-terminus of SecA

SecA is functional as a dimer, and the carboxy-termini of both monomers are in close proximity (Driessen, 1993). To investigate if both carboxy-termini are necessary for SecB binding, heterodimers of SecA and SecAN880 were formed by reversible unfolding and refolding. Wild-type SecA, SecAN880 and an equimolar amount of both proteins were mixed in the absence or presence of 6 M GdnHCl, and subsequently refolded by dilution. By this treatment, SecA can be refolded in a functional state (Driessen, 1993; Breukink *et al.*, 1995; data not shown). The [^{125}I]SecB-binding activity of the refolded and native wild-type SecA are nearly indistinguishable (Figure 5), while both the refolded and native forms of SecAN880 are defective in SecB binding. As expected, the SecB-binding activity of an equimolar mixture of native or separately refolded wild-type SecA and SecAN880 was ~50% of the activity found for wild-type SecA. However, a refolded equimolar mixture of wild-type SecA and

SecAN880 showed ~20% of the SecB-binding activity found for the refolded wild-type SecA. These data demonstrate that both carboxy-termini of the SecA dimer are needed for high-affinity SecB binding.

SecB is released from SecA at an early stage during preprotein translocation

SecA-dependent binding of SecB to inner membranes is promoted by preproteins (Hartl *et al.*, 1990; Figure 4C). To determine whether this phenomenon is due to an increase in binding affinity (K_d) or number of binding sites (B_{max}), binding of [^{125}I]SecB to IMVs was analysed by Scatchard analysis in the absence and presence of proOmpA (Figure 6A and B, Table I). In the absence of proOmpA, SecA binds SecB with a K_d of ~30 nM and a B_{max} of 115 pmol/mg of membrane protein. The presence of proOmpA promotes SecB binding by increasing the affinity 3-fold, i.e. 10 nM, whereas the B_{max} remains unchanged. OmpA has no influence on the affinity or on the B_{max} (Hartl *et al.*, 1990; data not shown). The number of binding sites for SecB is in agreement with the number of binding sites for SecA (Table I, Figure 6C), suggesting that a stoichiometric complex of one dimeric SecA with one tetrameric SecB is formed at the membrane.

To study the timing of SecB dislocation from the translocase, SecB binding studies were performed under translocation conditions. At the temperature used for translocation, i.e. 37°C, SecA binds SecB with a lower affinity than at 0°C, both in the absence and presence of precursor protein (Table I), while the number of binding sites remains unchanged. IMVs with bound SecA, SecB and proOmpA were isolated, and the translocation of [^{35}S]proOmpA was initiated by the addition of 2 mM ATP. In a parallel experiment, the release of [^{125}I]SecB from the IMVs was followed. The disengagement of SecB from the translocase was not coupled to the appearance of fully translocated proOmpA (Figure 7A). This suggests that the release of SecB from the translocation complex occurs at a very early stage in translocation. To substantiate this conclusion, the effect of ATP on the SecA-dependent SecB binding to IMVs was studied. Both in the absence and presence of precursor protein, ATP reduced the SecB binding severely at 0°C as well as 37°C (Figure 7B). Since translocation only takes place at 37°C, it seems that the SecB release is not coupled to translocation *per se*. SecB release is induced by binding of ATP, as similar effects were observed when the non-hydrolysable ATP analogue AMP-PNP was used (data not shown). Scatchard analysis of the SecB binding at 0°C in the presence of proOmpA and ATP revealed that the SecB release is due to a reduction in the number of binding sites rather than a change in K_d (Figure 8).

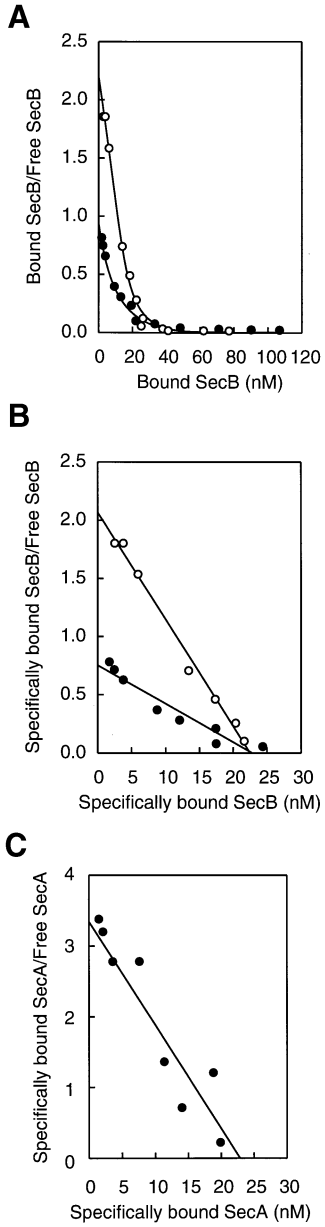

Fig. 6. ProOmpA promotes SecB binding to membrane-bound SecA by elevating the affinity of interaction. (**A**) Scatchard plot analysis of the binding of [^{125}I]SecB (4 nM to 5 μM) to urea-treated membranes derived from SecYEG$^+$ cells in the presence of 50 μg/ml (250 nM) SecA, and in the presence (○) or absence (●) of proOmpA. (**B**) Scatchard plot analysis of specific binding of SecB in the presence (○) or absence (●) of proOmpA. The fraction of non-specific SecB binding was 0.02. (**C**) Scatchard plot analysis of the specific membrane binding of [^{125}I]SecA (1–600 nM). The fraction of non-specific SecA binding was 0.04. Binding experiments were performed as described in the legend to Figure 3.

Discussion

SecB functions as a coupling factor that binds to nascent secretory proteins when they emerge from the ribosome (Kumamoto and Francetić, 1993; Randall *et al.*, 1997). It stabilizes them in an unfolded, translocation-competent conformation (Lecker *et al.*, 1989), and targets them to the SecA subunit of the translocase (Hartl *et al.*, 1990). Deletion analysis of SecA has shown that the carboxy-terminal 70 amino acids of SecA are not required for *in vitro* translocation (Matsuyama *et al.*, 1990), but that this region of SecA is needed for the viability of *E.coli* cells (Breukink *et al.*, 1995). The removal of this region interferes with the ability of SecA to interact with SecB, suggesting that the carboxy-terminal domain of SecA may contain the SecB-binding site (Breukink *et al.*, 1995). Since the evidence was based mainly on deletion analysis, we have now performed a reciprocal experiment, and fused various carboxy-terminal parts of SecA to GST. These GST fusion proteins genuinely bind SecB or a SecB–

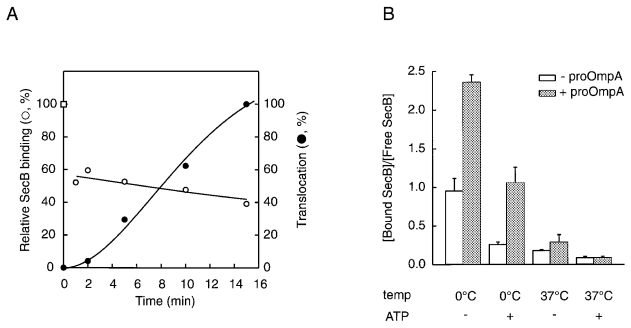

Fig. 7. SecB is released in an early step in translocation. (**A**) Time-dependent translocation of proOmpA and release of SecB. Pre-formed complexes of 10 nM [^{125}I]SecB, 300 nM [^{35}S]proOmpA, 250 nM SecA and urea-treated membranes of SecYEG^{+} cells were incubated for 15 min at 0°C and isolated via centrifugation through a 0.2 M sucrose cushion. The pellet was resuspended and, after pre-incubation for 2 min at 37°C, 2 mM ATP was added to initiate the translocation reaction (t = 0). The release of SecB (○) and translocation of proOmpA (●) was followed for 15 min. The amount of SecB bound to membranes at t = 0 (no ATP, □) and the amount of fully translocated (pro)OmpA at t = 15 min were set to 100%. (**B**) SecA-dependent SecB binding to membranes is reduced in the presence of ATP. SecA-dependent SecB binding to urea-treated membranes of SecYEG-overproducing cells was assayed as indicated in the absence (white bars) and presence (grey bars) of 300 nM proOmpA at 0 and 37°C, and in the absence or presence of 2 mM ATP.

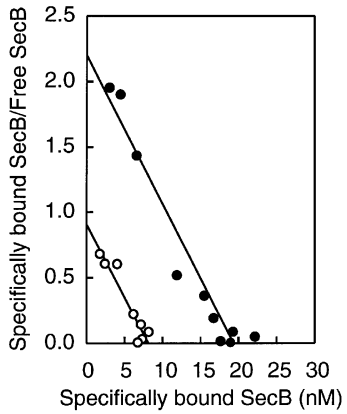

Fig. 8. ATP binding to SecA reduces the number of SecB high-affinity membrane-binding sites. [^{125}I]SecB binding to urea-treated membranes derived from SecYEG^{+} cells was determined in the presence (○) or absence (●) of 2 mM ATP as described in the legend to Figure 6. The fraction of non-specific SecB binding was 0.015.

preprotein complex provided that the extreme carboxy-terminal 22 aminoacyl residues of SecA are present. Moreover, these fusion proteins compete with the SecYEG-bound SecA for SecB binding. A moderate deletion of only the carboxy-terminal 21 aminoacyl residues of SecA results in a protein that is fully active in translocation, but that is unable to interact with SecB. These studies therefore identify a surprisingly short polypeptide stretch of SecA as the authentic SecB-binding site, and demonstrate that in the ternary SecA–SecB–precursor protein complex, the precursor protein is either bound to SecB or to SecA, but never to both proteins at the same time. The latter is evident from the observations that even in the presence of precursor protein, the SecAN880 does not bind SecB, and, additionally, that OmpA fails to bind SecA, regardless of the presence of SecB.

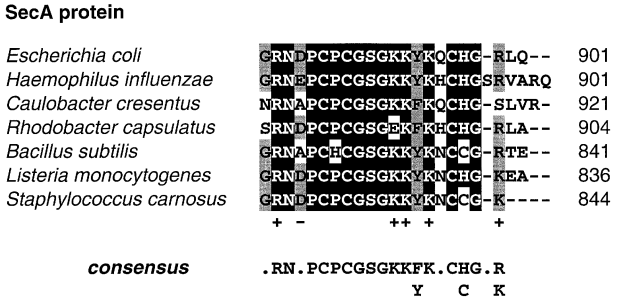

Fig. 9. SecB-binding domain of the bacterial SecA proteins is highly conserved. Alignment of the carboxy-termini of SecA proteins of *Escherichia coli*, *Haemophilus influenzae*, *Caulobacter cresentus*, *Bacillus subtilis*, *Listeria monocytogenes*, *Staphylococcus carnosum* and *Rhodobacter capsulatus.* Perfectly and well-conserved residues are black- and grey-boxed, respectively. Positively- and negatively-charged amino acid residues are indicated by + and –, respectively. The dot in the consensus sequence of the SecB-binding motif stands for any amino acid residue.

With the exception of *Streptomyces*, *Mycobacterium* and *Mycoplasma* species, the carboxy-terminus of SecA is highly conserved among the bacterial SecA proteins (Figure 9) with the consensus sequence: [GN]RNxP-C[PH]CGSGKK[YF]K[NQ]C[CH]G. We therefore propose this sequence as the consensus SecB-binding motif. Due to the high content of glycine and proline residues, this polypeptide stretch is likely to be highly flexible. The presence of lysyl (plus arginyl and histidyl) residues will give this domain a strong electropositive surface with predicted pI values of 9.5–10.8. SecB interacts with low affinity with peptides that carry a net positive charge (Randall, 1992), most of which are also membrane-surface active. As suggested by Breukink *et al.* (1995), this phenomenon most likely reflects the SecB–SecA interaction, rather than the SecB–preprotein interaction. In this respect, the observed lipid-interacting ability of the carboxy-terminus of SecA (Breukink *et al.*, 1995) may well be due to its charged properties. It should be emphasized that the SecA–SecB interaction is highly specific, and not merely determined by electrostatics. Studies by Rajapandi and Oliver (1994) suggest that serine substitution at the conserved C896 and C885 or C887 in the SecB-binding region of SecA already compromise its translocation activity. These cysteines do not need to be in an oxidized state, i.e. as disulfide bridges, since the SecA–SecB interaction at the membrane is not affected by the redox state of the solution, i.e. reduced [in the presence of dithiothreitol (DTT)] or oxidized (in the presence of sodium tetrathionate) (P.Fekkes, unpublished results). Since the consensus SecB-binding motif is not present in the cyanobacterial, plastid and chloroplast SecA homologues, it seems that these organisms and organelles lack a classic SecB homologue.

When SecB is complexed to proOmpA, it still recognizes the SecB-binding site of the GST–SecA fusion protein. This indicates that the SecA-binding site on SecB differs from the preprotein-binding site. Mutations in the presumed preprotein-binding site of SecB that cause translocation defects can be divided into two classes (Gannon and Kumamoto, 1993; Kimsey *et al.*, 1995). The first class of mutants is characterized by a reduced ability to form stable complexes with the precursor form of

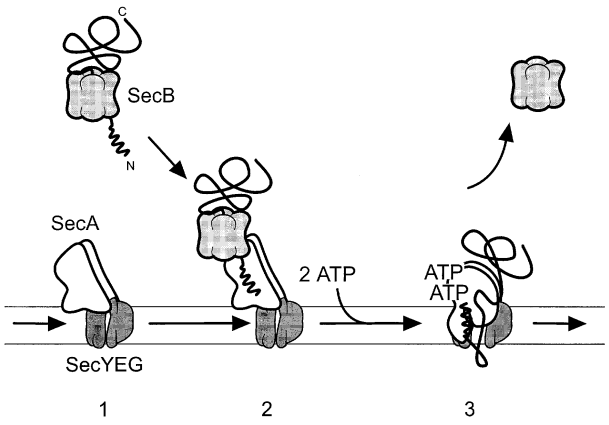

Fig. 10. Model for early steps in precursor protein translocation. See Discussion for details.

maltose-binding protein (preMBP), but causes only mild defects in the rate of preMBP export. The pattern of mutations suggests that the primary preprotein-binding site is hydrophobic and presumably contains β-sheet secondary structure. A hydrophobic character of the preprotein-binding site was also evident from the fluorescence characteristics of the fluorophore acrylodan that was derivatized to SecB-bound model polypeptides (Fekkes *et al.*, 1995). The second class of mutants is characterized by a severe translocation defect, but does not disrupt the SecB–preMBP complex formation. These mutations are confined to a single region and are largely in acidic residues. These residues are conserved in the SecB homologues of *Haemophilus influenzae* (Fleischmann *et al.*, 1995) and *Buchnera aphidicola* (Lai and Baumann, 1992) that share an overall identity of 36 and 47% with the *E.coli* SecB, respectively. Given the nature of these residues and their alternating appearance in the SecB domain, one may hypothesize that this region forms a β-structured acidic bristle that interacts with the electropositive carboxy-terminus of SecA. Recent evidence indicates that these mutants are indeed defective in SecA binding (P.Fekkes, J.G.de Wit, H.H.Kimsey, C.A. Kumamoto and A.J.M.Driessen, unpublished results).

Cytosolic SecA binds SecB with an almost 50-fold lowered affinity as compared with the membrane-bound SecA, i.e. >1.5 μM versus 30 nM, respectively (Den Blaauwen *et al.*, 1997). This implies that SecA that is peripherally associated with the SecYEG protein is primed for SecB binding and, for this purpose, exposes the carboxy-terminus. Addition of ATP results in a substantial dislocation of the SecB from the membrane, which may be caused by the large conformational change of SecA upon binding of ATP (Den Blaauwen *et al.*, 1996). Unlike many other chaperones, SecB itself is not an ATPase. Our data demonstrate that SecB utilizes the ATP-binding capacity of SecA for the active release from the translocase.

Based on the above considerations, we propose the following cascade of events during the initial stages of precursor protein translocation (Figure 10): (i) the SecYEG-bound form of SecA binds the binary SecB–precursor protein complex with high affinity through the recognition of SecB; (ii) SecB donates the precursor protein to SecA, and remains bound to the carboxy-terminus of SecA; (iii) binding of ATP to SecA elicits a large conformational change that disengages SecB, causes SecA to release the signal sequence domain of the bound precursor to the SecYEG complex and drives the membrane integration of SecA and the translocation of a precursor protein segment by co-insertion. The released SecB then will bind a cytosolic precursor protein or nascent chain before it re-enters the translocation cascade.

In conclusion, SecB fulfils an important role in targeting the precursor protein to the translocase by binding the carboxy-termini of the SecA dimer. The SecB–translocase interaction is synchronized with the catalytic cycle of SecA in that SecB uses the ATP-binding capacity of SecA to get removed from the translocation site. Once the precursor protein has been delivered to SecA, and translocation is initiated by the binding of ATP, the membrane-bound form of SecB becomes obsolete and is released into the cytosol to rebind another precursor protein.

Materials and methods

Bacterial strains and growth conditions

All strains used were *E.coli* K12 derivatives. Cloning experiments were performed in DH5α (Hanahan, 1983). Overexpression of the SecYEG complex was in strain SF100 [*F*⁻ Δ*lacX74 galE galK thi rpsL (strA)* Δ*phoA(PvuII)*, Δ*ompT*] (Baneyx and Georgiou, 1989) harbouring plasmid pET340 (Van der Does *et al.*, 1996). Overexpression of GST and GST fusion proteins was done in strain NO2947 (Δ*lac[IPOZYA], araD139*,Δ*[ara-leu]7697, galU, galK, rpsL, recA56 srl*::Tn*10*, $r_k m_k$). Unless indicated otherwise, strains were grown aerobically at 37°C on LB-broth supplemented with 50 μg of ampicillin/ml.

Materials

SecA (Cabelli *et al.*, 1988), SecB (Weiss *et al.*, 1988), proOmpA and OmpA (Crooke *et al.*, 1988) were purified as described. SecA and SecB were labelled with carrier-free ^{125}I (Radiochemical Centre, Amersham, UK) to specific activities of ~2×10^6 and 1.6×10^6 c.p.m./μg, respectively. Proteins (100 μg) were suspended in 200 μl of buffer A (50 mM Tris–HCl pH 7.6, 50 mM KCl, 5 mM $MgCl_2$), and transferred to a reaction vial coated with IODO-GEN Iodination Reagent (Pierce, Rockford, IL). Reactions were started by adding 2 μl of $K^{125}I$ (200 μCi), incubated for 15 min on ice, and terminated by transferring the mixture into a new reaction vial containing 10 mM DTT. Free iodine was removed by chromatography on a PD-10 Sephadex column (Pharmacia Biotech AB, Uppsala, Sweden) which was pre-washed with buffer A containing 1 mM DTT. Inverted IMVs were prepared from *E.coli* SF100 as described by Chang *et al.* (1978), and treated with 6 M urea (Cunningham *et al.*, 1989). Polyclonal antiserum (pAb) 1045 (Neosystem Laboratoire, Strasbourg, France) was raised against a synthetic peptide H_2N-GRNDPSPCGSGKKYKQCHGR-COOH which corresponds to amino acids 880–899 of SecA, with the exception that Cys885 is replaced by Ser (Van der Does *et al.*, 1996).

Construction and purification of the GST fusion proteins

PCR reactions were performed with pMKL18 (Klose *et al.*, 1993) as template and the primer combinations for809 and revEND, for809 and rev879, and for880 and revEND (Table II) to obtain fragments coding for different parts of the carboxy-terminus of SecA. PCR fragments were digested with *Eco*RI and *Bam*HI and ligated into pSKII$^+$ (Stratagene, La Jolla, CA), resulting in plasmids pET213, pET214 and pET215, respectively. The nucleotide sequence of the inserts was verified by automatic sequencing on a Vistra DNA sequencer 725 (Amersham, Buckinghamshire, UK). pET213, pET214 and pET215 were digested with *Eco*RI and *Bam*HI and inserts were ligated into pGEX-4T1 (Pharmacia), yielding pET216, pET217 and pET218, respectively. For the purification of the GST fusion proteins, exponentially growing cells were supplemented with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at OD_{660} ~0.5 and grown for another 2 h. Cells were harvested by centrifugation (10 000 *g*, 10 min) and lysed by French pressure treatment (two passes at 900 p.s.i.). The cleared lysate (40 000 *g*, 15 min) was incubated with glutathione-conjugated Sepharose 4G beads and the GST fusion proteins were isolated according to the manufacturer's notes (Pharmacia).

Table II. PCR primers used to construct GST–SecA fusion proteins and SecAN880-truncate

Primer	Sequence	Introduced features
for809	5′-CGGGATCCATGTTTGCAGCGATGCTG-3′	*Bam*HI site
for880	5′-CGGGATCCGGACGTAACGATCCTTGCCCG-3′	*Bam*HI site
rev879	5′-TGAATTCTTATACTTTGCGCTCTCCGG-3′	*Eco*RI site, stop codon at position 880
revEND	5′-TGAATTCCGCAGAATCCTGCC-3′	*Eco*RI site
for785	5′-CGGGATCCTGCGTGGCTACGC-3′	*Bam*HI site
rev880	5′-CGGAATTCTTATTATCCTACTTTGCGCTC-3′	*Eco*RI site, stop codon at position 881

Construction of the SecAN880

To introduce a stop codon at position 881 of SecA, a PCR reaction was performed with primers for785 and rev880 and pMKL18 as template. The resulting fragment was digested with *Eco*RI and *Bam*HI and ligated into pSKII$^+$ digested with the same enzymes to obtain pSK880. After verification of the correctness of the insert by sequencing, pSK880 was digested with *Sna*BI and *Eco*RI and the 153 bp fragment was ligated into pUC19, in which the 1282 bp *Kpn*I–*Nde*I fragment of pMKL18 was cloned. The resulting plasmid was then digested with *Kpn*I and *Nde*I and the fragment coding for the truncated carboxy-terminus of SecA was ligated into pMKL18, resulting in pET259.

Binding studies

Purified GST fusion protein and SecB were pre-incubated at room temperature for 10 min in 100 µl of buffer C (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3). Subsequently, 10 µl of buffer C-washed glutathione-conjugated Sepharose 4B beads were added and, after 10 min, the mixture was transferred to a quick-spin column (Promega, Madison, WI) and centrifuged for 5 min at 5000 r.p.m. The column was washed twice with 200 µl of buffer C, and GST was eluted by a repeated wash with 50 µl of 10 mM reduced glutathione in buffer C. Eluted samples were pooled and analysed by SDS–PAGE (Laemmli, 1970) and blotted on PVDF membranes (Millipore, Bedford, MA) using a semi-dry blotter (Bio-Rad, Hercules, CA). Immunodetection was carried out with pAb raised against SecB. Blots were developed with the chemiluminescence kit (Tropix, Bedford, MA).

Binding of ^{125}I-labelled SecA and SecB to urea-treated inner membranes was performed as described (Hartl *et al.*, 1990). Molar concentrations of SecA and SecB are calculated assuming that SecA is a dimer and SecB is a tetramer, respectively.

In vitro translocation of ProOmpA

In vitro translocation of ^{35}S-labelled proOmpA into IMVs of *E.coli* was assayed by its accessibility to added proteinase K (Cunningham *et al.*, 1989). ^{35}S-labelled proOmpA was obtained with an *in vitro* transcription/translation reaction (De Vrije *et al.*, 1987), and affinity-purified as described (Crooke and Wickner, 1987). Reaction mixtures (50 µl) contained: buffer B [50 mM HEPES–KOH, pH 7.5, 30 mM KCl, 0.5 mg/ml bovine serum albumin (BSA), 10 mM creatine phosphate, 5 µg/ml creatine kinase, 10 mM DTT and 2 mM $Mg(OAc)_2$], 20 µg/ml SecA (100 nM), 32 µg/ml SecB (500 nM), 1 µl of urea-denatured [^{35}S]proOmpA and IMVs (15 µg of protein). This mixture was pre-incubated for 5 min at 37°C, and the reactions were initiated by the addition of 2 mM ATP. Samples were treated with proteinase K (0.1 mg/ml) for 15 min on ice, precipitated with 7.5% (w/v) trichloroacetic aciid, washed with ice cold acetone, solubilized in SDS sample buffer and analysed by 12% SDS–PAGE (Laemmli, 1970) and autoradiography.

Other techniques

The ATPase activity of SecA and SecAN880 was assayed using the method of Schiebel *et al.* (1991). Protein determination was performed according to Bradford (1976) or Lowry *et al.* (1951) with BSA as standard. The concentrations of SecA and SecB were determined by amino acid content analysis (Eurosequence, Groningen, The Netherlands).

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